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Complete nucleotide sequence of the influenza B/Singapore/222/79 virus hemagglutinin gene and comparison with the B/Lee/40 hemagglutinin

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ABSTRACT
    The complete nucleotide sequence of the hemagglutinin (HA) gene of the human type B influenza virus B/Singapore/222/79 is presented. Comparison with the only other known sequence of a B hemagglutinin (B/Lee/40) shows that antigenic drift in type B HA genes is essentially the same as already observed within the influenza A H3 subtype, i.e., an accumulation of point mutations. The main difference is that the apparent evolution is significantly slower, most likely due to the cumulative effect of a lower occurrence in the population (slower evolution) and/or less immunological pressure. There is a striking cluster of changes at positions 127 until 137 of the HA1 subunit which may represent one of the antigenic sites of the molecule.

INTRODUCTION
    Antigenic variation in influenza A viruses has been studied extensively both for the hemagglutinin (1,2) and more recently for the neuraminidase (3-10). For influenza B, however, much less information about its surface antigens is available, with only the structure of one hemagglutinin gene (11) and one neuraminidase gene (12), both from influenza B/Lee/40, having been published so far. Antigenic shift has not been observed in type B viruses which may be due to the absence of a gene pool of influenza B viruses in animals (13). Antigenic drift, however, has been shown to occur, but at a slower rate than in influenza A (14,15). Studies with monoclonal antibodies, directed against the HA of B/Lee/40 virus also indicated that the B type HA is less variable than the A type HA (16,17). Comparison of the influenza B/Lee/40 HA with the type A hemagglutinins shows a remarkable conservation of the major structural features of the HAs of both type A and B viruses, suggesting a close evolutionary relationship of the corresponding genes (11).
    In this paper, we present the complete nucleotide sequence of the HA gene of B/Singapore/222/79 virus. This virus strain is present in influenza vaccines for use during the 1983-1984 season, as recommended by the World Health Organization (18).

MATERIALS AND METHODS

Viral and bacterial strains

The influenza strain B/Singapore/222/79 was a generous gift of the companies Duphar (Weesp, Holland) and Mérieux (Marcy l'Etoile, France). The viral RNA was extracted according to the procedure of Palese and Schulman (19). Plasmid pBR322 derivatives, containing viral DNA inserts, were propagated in E. coli DH1 cells (20,21).

Construction of recombinant plasmids containing influenza genes

The equimolar mixture of 8 viral RNAs (~30 μg) was 3'-polyadenylated and converted to single stranded DNA copies with reverse transcriptase followed by RNAase treatment as described previously (22). Second strand synthesis was initiated with the dodecanucleotide primer d(AGTAGAAACAAG) (Collaborative Research, Inc., Waltham, Mass.). The cDNA and the primer (5 μg) were dissolved in 25 μl 80mM K_2HPO_4 (pH 6.9), boiled for 30 sec and left to cool slowly to room temperature. The reaction mixture was diluted to 50 μl by the addition of dithiotreitol (4 mM final concentration), $MgCl_2$ (6 mM), K_2HPO_4 (140 mM), dNTPs (300 μM), 50 μCi [α-^{32}P]-dATP and 15 U DNA polymerase I (Boehringer, Mannheim), and kept at 15°C for 3 h. The dsDNA was purified by extraction with phenol/chloroform/isoamylalcohol (25:24:1) followed by gel filtration on a G50 fine Sephadex column and precipitated with isopropanol. dsDNA copies of the HA and NP gene were isolated after fractionation of the crude mixture on a 1.5% agarose gel and cloned into the Pst I site of pBR322 using the poly(dG)-poly(dC) tailing method (23).

Identification of HA specific inserts

Tetracycline and ampicilline sensitive colonies were screened for HA and NP gene inserts using colony hybridization with a [^{32}P](HA + NP)-RNA probe as described previously (22). Positive clones were grouped by restriction analysis and conclusively identified by sequence analysis of both ends. However, the longest HA insert found lacked some information. A suitable ^{32}P-labeled DNA restriction fragment located near the missing end was used as a probe to rescreen our collection of HA and NP colonies essentially following the procedure of Hanahan and Meselson (24).

Nucleotide sequence analysis of the HA gene

Sequence analysis was performed according to the procedures of Maxam and Gilbert (for dG, dA, dC and dC + dT) (25) and Peattie (dG + dA) (26). We used 0.25 mm x 60 cm sequencing gels, essentially prepared as described by Ansorge and De Maeyer (27) and Garoff & Ansorge (28).

RESULTS AND DISCUSSION

Cloning and characterization of HA gene inserts

Synthesis of the second strand of a cDNA according to common standard procedures makes use of the self-priming capacity of the cDNA, followed by S1 nuclease treatment. It is inevitably coupled with the loss of some

information corresponding to the 5'-end of the RNA. For cloning influenza viral RNAs, there is an additional problem for first strand synthesis because of its lack of a 3' poly A-tail. It is possible to polyadenylate the viral RNA in vitro before first strand DNA synthesis (22). For influenza A strains, both problems are overcome by the use of two (commercially available) universal oligonucleotides to prime reverse transcription (avoiding polyadenylation) and to initiate second strand DNA synthesis (29-31). Lacking a suitable primer to initiate reverse transcription for influenza B, we synthesized a ssDNA copy of the HA gene in the conventional way using polyadenylation and oligo-(dT) priming (22). Second strand synthesis, however, was primed with the dodecamer d(AGTAGAAACAAG). As the HA and NP genes of influenza B viruses are nearly of the same size (32), we ended up with a mixture of clones containing dsDNA copies of both genes, and the HA specific clones were identified by sequence analysis of the terminal regions. Clone pSHA2 had the complete 3'-sequence information (starting with the primer) but was incomplete at the 5' terminus. A nick-translated ^{32}P-labeled Hind III - Mbo I restriction fragment positioned close to the 5' coding end of the pSHA2 insert was used as a probe for colony hybridization. Several clones containing the missing 93 nucleotides were detected and sequenced (pSHA38, pSHA63 and pSHA68).

Nucleotide sequence

The nucleotide sequence of the influenza B/Singapore HA gene is shown in Fig. 1. The entire viral RNA contains 1878 nucleotides and has the coding capacity for a precursor polypeptide of 583 amino acids. At the 5' end of the coding strand, a non-translated region of 33 nucleotides occurs, while the 3' end shows an untranslated sequence of 96 residues.

Protein sequence

No protein sequence data on the B/Singapore/222/79 HA is available but the amino acid terminal sequences of the HA1 and HA2 subunits (33) and, very recently, the nucleotide sequence of the B/Lee/40 HA (11) have been determined. Comparison of the B/Singapore with the B/Lee HA (Fig. 1) shows a high degree of homology and suggests the same build-up of the molecule. The HA precursor starts with a 15 residue signal sequence, followed by an HA1 chain consisting of a maximum of 345 amino acids, and an HA2 peptide composed of 223 residues. The HA1 subunit may undergo further processing by a carboxy-peptidase B-like enzyme and/or a trypsin-like enzyme (34).

Comparison of influenza B HAs

A comparison of the nucleotide and amino acid sequences of the HA of B/Singapore/222/79 and B/Lee/40 virus is shown in Fig. 1. The B/Lee gene contains an extra AAC triplet in the HA1 peptide (coding for amino acid 165) and one extra G residue in the 3' untranslated region. An extra codon insertion (also AAC) was first observed by us in a comparison of A/Victoria/3/75 with A/Aichi/2/68 HA (35). Similar insertions (deletions)

of AAC or ACA triplets were found in several influenza A HA (35-37) and NA genes (38), while AAA insertions were observed in laboratory variant strains of the A/PR8/34 HA gene (39). In all these cases, the extra codon was located in a very U + G-rich region of the (complementary) vRNA and for chronological reasons, it seems that insertion is a more likely explanation rather than a deletion. Insertion of a few nucleotides can readily be explained by slippage of the viral polymerase (38). Slippage of the reverse transcriptase during cDNA synthesis is another possible explanation. But, since this has to our knowledge never been found when other RNAs were converted to DNA using this enzyme and since the same insertion was found in independent clones (22), this possibility must be considered very unlikely. As the extra AAC-triplet is present in B/Lee/40, it seems at face value that a deletion is more likely. But, it should be noted that the B/Singapore/79 is not necessarily a direct descendant and therefore an insertion during the evolution from a common ancestor gene to B/Lee/40 is at least as probable.

The sequence comparison of two type B HAs shows the changes that have resulted after 39 years of evolution. The number of nucleotide substitutions is 133 (for the total gene) or 7.1% at the nucleotide level. These substitutions give rise to 36 amino acid changes or 6.2%.

In the 5'-untranslated region, the three clustered substitutions, GTT→AGA (nucleotides 10-12) are observed. This is very remarkable, as these substitutions occur in the region of the first 12 nucleotides of the 3' end of the vRNA, which is conserved to a high degree in all genes of the same type of influenza virus. The 3'-untranslated region of the B/Singapore HA shows 8 substitutions (the extra G in B/Lee/40 included) with one of them changing the nature of the termination codon (UGA→UAA). This is, therefore, a second difference with the variation in influenza A, where HA genes, belonging to the same subtype, have identical termination codons and 3' untranslated sequences (1,35). Another remarkable difference with the variation observed in influenza A viruses is the exact conservation of the signal sequence as not even one silent nucleotide substitution was observed. Possibly, this may even reflect a biological function at the RNA level in this region implying that this region must be

Figure 1: Comparison of the hemagglutinin of B/Singapore/222/79 and B/Lee/40 virus.
The complete nucleotide and predicted amino acid sequence of the B/Singapore HA is shown. The B/Lee numbering is used. Only changes in nucleotide sequence (underneath) and amino acid sequence (above) are indicated for the B/Lee HA. The post-translational cleavage sites, where the pre-HA is cleaved into a signal peptide (15 amino acids), an HA1 peptide (maximum 346 amino acids) and an HA2 peptide (223 amino acids) are indicated by arrows. Potential glycosylation sites are boxed. The site at residue 165 of HA1 is absent in B/Lee (see text).

Table I

Evolution of B Type HAs Compared with the Evolution of A type HAs

			% substitutions in B type / % substitutions in A type
HA1	% nucleotide substitutions	B type : 2.0 A type : 5.7	0.35
	% amino acid substitutions	B type : 2.0 A type : 9.1	0.22

Table 1: Only the HA1 part has been taken into account. The evolution of the B type was calculated for the HA1 regions of B/Lee/40 and B/Singapore/79. For the A-type data, A/Aichi/68 (35) and A/Bangkok/79 (40) were used. The observed percentages were recalculated for a period of 10 years.

preserved in influenza B viruses.

As in influenza A HAs, the HA1 peptide is the most variable part of the molecule. The HA1 subunit shows 7.8% nucleotide substitutions, giving rise to 8.7% amino acid changes. For the HA2 region, these values amount to only 6.1% and 2.7%, respectively. As we have explained before (35), the occurrence of mutations in the two parts of the gene is undoubtedly of similar frequency, but many more mutants in the HA2 part are selected away because this domain fulfills an enzyme-like activity and the structure-function constraint is very tight. Mutants in the HA1 part, on the other hand, occasionally confer a selective advantage in overriding immunological barriers. Except for the extra triplet and an extra G-residue (in the 3'-untranslated region), all substitutions can be explained by point mutations. Our data shows that in general, the antigenic drift observed in influenza B HAs is comparable with the drift observed in influenza A HAs belonging to the H3 subtype (2,35). The apparent rate of evolution, however, is significantly slower, especially at the protein level. Comparing the drift between A/Aichi/2/68 (35) and A/Bangkok/79 (40) with that between B/Lee/40 and B/Singapore/79, we calculate for the B virus HA1 that nucleotide substitutions occurred three times slower and amino acid mutations occurred four times slower than in their A-type counterparts (see Table 1). These data confirm and extend previous studies on influenza B virus strains with monoclonal antibodies which had suggested a lower variability (16,17).

Glycosylation

The pattern of potential N-glycosylation sites varies slightly between the type B HAs (Fig. 1). The B/Singapore HA shows eleven potential carbohydrate attachment sites (one more than B/Lee/40 at position 145 of the HA1 peptide). This is not unusual as type A HAs, belonging to the same subtype, also show slightly variable glycosylation patterns (1,35). For the influenza B HAs, it is not known which of these potential sites have, indeed, carbohydrates attached. Influenza B HAs show 4 potential glycosylation sites at the C-terminus of their HA2 peptides, in contrast to influenza A HAs which have, at most, 2 potential carbohydrate attachment sites on their HA2 molecules (1,35). However, it seems highly unlikely that there is a 4th carbohydrate unit attached to residue 216 of the HA2 since it is presumably located in the tail region inside the virion.

A possible antigenic site in influenza B HAs

Studies with monoclonal antibodies suggest that the B type HA has at least three partially overlapping antigenic determinants (17).

Fig. 1 shows a very unequal distribution of the amino acid substitutions over the molecule. Two thirds of the amino acid changes are located in a region covering only 25% of the total HA1 chain. The most striking group of substitutions occurs in the cluster from residue 127 until 137. Three out of six changes in the cluster are infrequently found in related protein pairs: Thr→Arg, Arg→Gly and Lys→Ile (41). The amino acids are more conserved on both sides of this cluster. This is strongly reminiscent of the structure of the proposed antigenic sites of the Hong Kong HA (42) and suggests this cluster to be one of the predominant antigenic determinants of the type B HA.

The studies of Krystal et al. (11) revealed extensive structural similarities between influenza A and B HAs e.g., a nearly complete conservation of cysteine residues. Comparison of the B/Singapore and A/Aichi HA (35) sequence confirms these data and suggests a very similar 3-dimensional structure for A and B type HAs. Alignment of the B/Singapore and A/Aichi HA sequences (Fig. 2) shows that the region containing residues 127 until 137 of the B/Singapore HA1 corresponds to the region 123 until 133 of the A/Aichi HA1. This peptide segment occurs on a surface loop of the globular part of the molecule, close to and presumably part of the antigenic site A of the H3 HA and may play a similar role in B viruses. It is also evident from Fig. 2 that the hydrophilic stop-transfer sequence near the carboxy-terminus and the short hydrophilic tail are in exactly the same positions in A and B viruses.

Concluding remarks

Antigenic variation in influenza B HAs does not seem to differ in essential aspects from the drift observed within an A subtype HA. However, the apparent rate of evolution proceeds significantly slower. Most likely,

HA1

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                 ↓    *10        20  *    30        *40                               50         60        70
A/Aichi/68   QDLPGNDNSTATLCLGHHAVPNGTLVKTIIDDQIEVTNATELVQSSST-----------GKICNNPHRILDGIDCTLITALLG
B/Sing /79   DRI----------CTGITSSNSPHVVKTATQGEVNVTGVIPLTTTPTKSHFANLKGTKTRGKLC------PNCLNCTDLDVALG
                        ■ ■      ■■ ■      *■ ■    ■                                    ■■ ■      *■    ■
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      80*         90        100       110       120                       130       140       150       160
DPHCDVFQNETWDLFV-ERSKAFSNCYPYDVPDYASLRSLVASSGTLEFIT--------EGFTWTGVTQNGGSNAC-KRGPGSGFFSRLNWLT-KSGSTY
RPKCMGTIPSAKASILHEVKPVTSGGFPIMHDRTK-IRQLPNLLRGYENIRLSTRNVINAERAPGGPYIIGTSGSCPNVTNGNGFFATMAWAVPKDNKTA
■■         ■       ■     ■        ■■     ■■             ■   ■  ■■■■ *  ■  ■■■     ■   ■  *■■
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    170       180       190            200       210       220       230       240       250
--PVLN-VTMPNNDNFDKLYIWGIHHPSTNQEQTSLYW----QA--SGRVTVST-RRSQQTIIPNIGSRPWVRGLSSRISIYWTIVKPGDVLVINS-NGN
TNPLTVEVPYICTKGEDQITVWGFHSD-TETQMVKLYGDSKPQKFTSSANGVTTHYVSQIGGFPN-QTEDGGLPQSGRIVVDYMVQKPGKTGTIVYQRGV
■    ■     ■  ■ ■■ ■     ■■■     ■   ■  ■■ ■     ‡              ■■ ■■     ■       ■      ■
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HA2

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    260       270       280       *290       300       310       320          ↓       340
LIAP-RGYF-KMRTGKSSIMRSDAPIDTCISE-CITPNGSIPNDKP-FQNVNKITYGACPKYVKQNTLKLATGMRNVP-EKQT--RGLFGAIAGFIENGW
LL-PQKVWCASGRSKVIKGSLPLIGEADCLHEKYGGLNKS----KPYYTGEHAKAIGNCPIWVKTP-LKLANGTKYRPPAKLLKERGFFGAIAGFLEGGW
■ ■       ■           ■ ■     ‡■ ■      ■            ■■ ■   ■■■ *■    ■  ■      ■ ■  ■■■
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    350       360       370       380       390       400       410       420       430       440
EGMIDGWYGFRHQNSEGTGQAADLKSTQAAIDQINGKLNRVIEKTNEKFHQIEKEFSEVEGRIQDLEKYVEDTKIDLWSYNAELLVALENQHTIDLTDSE
EGMIAGWHGYTSHGAHGVAVAADLKSTQEAINKITKNLNSLSELEVKNLQRLSGAMDELHNEILELDEKVDDLRADTISSQIELAVLLSNEGIINSEDEH
■■ ■ ■       ■         ■■■■■    ■        ■■ ■         ■     ■ ■   ■■ ■  ■  ■■ ■■ ■   ■
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    450       460       470       480       *490       500       510       520       530       540
MNKLFEKTRRQLRENAEEMGNGCFKIYHKCDNACIESIRNGTYDHDVY---RDEALNNRFQIKGVELKSGYKDWILWISFAISCFLLCVVLLGFIMWACQ
LLALERKLKKMLGPSAVDIGNGCFETKHKCNQTCLDRIAAGTFNAGEFSLPTFDSLNITAASLNDDGLDNHT-ILLYYSTAASSLAVTLMIAIFIVYMVS
■ ■ ■    ■ ■      ■■        *■  ■  ■         ‡            ■         ■      ~~~~~~~~~~~~~~~~~~~~~~~~~~
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    550
RGNIRCNICI
RDNVSCSICL
■ ‡  ■ ■■
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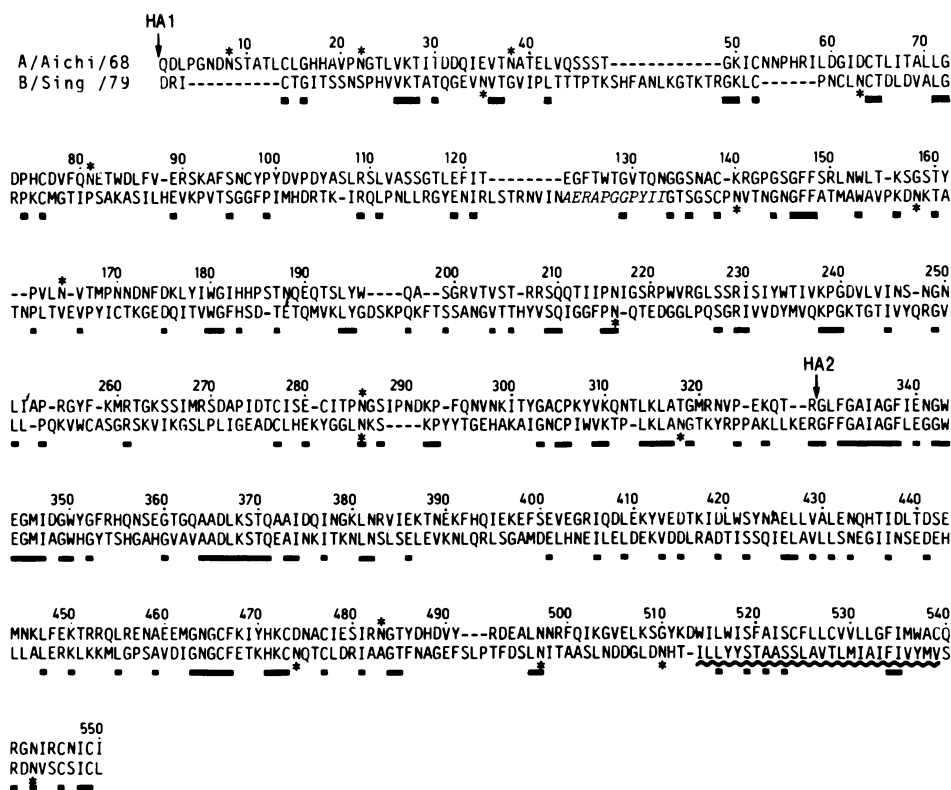
Figure 2: Comparison of the predicted amino acid sequences of the B/Singapore/222/79 and A/Aichi/2/68 virus mature HAs.
Alignment of the sequences was done on the basis of conserved amino acids between influenza A HAs. Homologous residues are underlined. Potential glycosylation sites are marked with an asterisk. The amino acids belonging to the proposed antigenic determinant of the B/Singapore HA are represented in italics. The Aichi numbering is used. The membrane spanning sequence is emphasized by a wavy line.

this can be explained as a combined effect of replication frequency (possibly the number of infection cycles) and immunological pressure. Influenza B is not as widespread in the human population ´as influenza A, consequently totalling less infection cycles and thereby reducing the total error frequency. Also, there are less antibody carriers which reduces the selection pressure for new antigenic variants. Many of the amino acid subsitutions(due to point mutations) appear as clusters in a limited region of the HAl subunit. The cluster containing residues 127-137 of the HAl may represent one of the antigenic determinants of the B type HA. Nevertheless, more data is required in order to obtain a detailed picture

of the antigenically important domains of influenza B HA.

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